TREATMENT OF ANEMIA USING TNFα INHIBITORS

Related Applications

This application claims priority to prior filed U.S. Provisional Application Serial No. 60/397,275, filed July 19, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/411,081, filed September 16, 2002, and prior-filed U.S. Provisional Application Serial No. 60/417490, filed October 10, 2002. This application also claims priority to prior filed to U.S. Provisional Application Serial No. 60/455777, filed March 18, 2003. In addition, this application is related to U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015. This application is also related to U.S. Patent Application Serial No. 09/801,185, filed March 7, 2001; U.S. Patent Application Serial No. 10/302356, filed November 22, 2002; U.S. Patent Application Serial No. 10/163657, filed June 2, 2002; and U.S. Patent Application Serial No. 10/133715, filed April 26, 2002.

This application is related to U.S. utility applications (Attorney Docket No. BPI-187) entitled "Treatment of TNFα-Related Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-188) entitled "Treatment of Spondyloarthropathies Using TNFα Inhibitors," (Attorney Docket No. BPI-189) entitled "Treatment of Pulmonary Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-190) entitled "Treatment of Coronary Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-191) entitled "Treatment of Metabolic Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-192) entitled "Treatment of Anemia Using TNFα Inhibitors," (Attorney Docket No. BPI-193) entitled "Treatment of Pain Using TNFα Inhibitors," (Attorney Docket No. BPI-194) entitled "Treatment of Hepatic Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-195) entitled "Treatment of Skin and Nail Disorders Using TNFα Inhibitors," (Attorney Docket No. BPI-196) entitled "Treatment of Vasculitides Using TNFα Inhibitors," (Attorney Docket No. BPI-197) entitled "Treatment of TNFα-Related Disorders Using TNFα Inhibitors," and PCT application (Attorney Docket No. BPI-187PC) entitled "Treatment of TNFα-Related Disorders," all of which are filed on even

date herewith. The entire contents of each of these patents and patent applications are hereby incorporated herein by reference.

Background of the Invention

The major function of red blood cells is to transport oxygen to tissues of the body. Minor functions include the transportation of nutrients, intercellular messages and cytokines, and the absorption of cellular metabolites. Anemia, or a loss of red blood cells or red blood cell capacity, can be grossly defined as a reduction in the ability of blood to transport oxygen. Anemia can be measured by determining a patient's red blood cell mass or hematocrit. Hematocrit values are indirect, but fairly accurate measures of the total hemoglobin concentration of a blood sample. Anemia, as measured by a reduced hematocrit, may be chronic or acute. Chronic anemia may be caused by extrinsic red blood cell abnormalities, intrinsic abnormalities or impaired production of red blood cells. Extrinsic or extra-corpuscular abnormalities include antibody-mediated disorders such as transfusion reactions and erythroblastosis, mechanical trauma to red cells such as micro-angiopathic hemolytic anemias, thrombotic thrombocytopenic purpura and disseminated intravascular coagulation. In addition, infections by parasites such as Plasmodium, chemical injuries from, for example, lead poisoning, and sequestration in the mononuclear system such as by hypersplenism can result in red blood cell disorders and deficiencies.

Impaired red blood cell production can occur by disturbing the proliferation and differentiation of the stem cells or committed cells. Some of the more common diseases of red cell production include aplastic anemia, hypoplastic anemia, pure red cell aplasia and anemia associated with renal failure or endocrine disorders. Disturbances of the proliferation and differentiation of erythroblasts include defects in DNA synthesis such as impaired utilization of vitamin B_{12} or folic acid and the megaloblastic anemias, defects in heme or globin synthesis, and anemias of unknown origins such as sideroblastic anemia, anemia associated with chronic infections such as malaria, trypanosomiasis, HIV, hepatitis virus or other viruses, and myelophthisic anemias caused by marrow deficiencies.

Cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) are molecules produced by a variety of cells, such as monocytes or macrophages, which

have been identified as mediators of inflammatory processes. TNFα (also referred to as TNF) is a cytokine produced by numerous cell types, including monocytes and macrophages, that was originally identified based on its capacity to induce the necrosis of certain mouse tumors (see *e.g.*, Old, L. (1985) *Science* 230:630-632). Cytokines regulate the intensity and duration of the inflammatory response which occurs as the result of an injury, disease, or infection.

Summary of the Invention

The invention includes provides methods for treating anemia where TNFα activity is detrimental in a safe and effective manner. Excessive or unregulated TNF production has been implicated in mediating or exacerbating a number of diseases including anemia (DeRienzo, *et al.* (1990) *Tex Med.* 86(10):80-3; Maury, CP *et al.* (1989) *Scan. J. Rheumatol.* 18(1):3-5; Means, RT Jr. (1997) *Cytokines Cell Mol. Ther.* 3(3):179-186).

In one aspect, the invention provides a method for treating a subject suffering from anemia, comprising administering to the subject a TNFα antibody such that the anemia is treated. In one embodiment, the TNFα antibody, or an antigen binding portion thereof, wherein the antibody dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less.

In another aspect, the invention provides a method for treating a subject suffering from anemia, comprising administering to the subject a TNFα antibody, or an antigen-binding portion wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6,

8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

In yet another aspect, the invention provides a method for treating a subject suffering from anemia in which TNFα activity is detrimental, comprising administering to the subject an antibody, wherein the antibody is an isolated human antibody, or an antigen-binding portion thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO:1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2. In one embodiment, the antibody or antigen binding portion thereof is D2E7, also referred to as HUMIRA® (adalimumab).

The invention also provides a method of inhibiting or reducing anemia in a subject comprising administering to the subject a therapeutically effective amount of a TNFα antibody, such that said anemia is inhibited or reduced. In one embodiment the TNFα antibody, or antigen binding portion thereof dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less. In one embodiment, TNFα antibody, or antigen binding portion thereof is D2E7.

In yet another aspect, the invention provides a method of treating anemia, wherein the TNFα antibody, or antigen–binding portion thereof, that dissociates from human TNFα with a K_d of 1 x 10^{-8} M or less and a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1 x 10^{-7} M or less. In one embodiment the antibody is a TNFα antibody, or an antigen-binding portion thereof, wherein the antibody dissociates from human TNFα with a K_{off} rate constant of 1 x 10^{-3} s^{-1} or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5,

6, 8, 9, 10, 11 and/or 12. In one embodiment the TNFα antibody, or antigen binding portion thereof is D2E7.

In a further another aspect, the invention provides a kit comprising a pharmaceutical composition comprising a TNFα antibody, or an antigen binding portion thereof, and a pharmaceutically acceptable carrier; and instructions for administering to a subject the TNFα antibody pharmaceutical composition for treating a subject who is suffering from anemia. In one embodiment, the TNFα antibody, or antigen binding portion thereof is D2E7.

Detailed Description of the Invention

This invention pertains to methods of treating anemia in which TNFα activity, *e.g.*, human TNFα activity, is detrimental. The methods include administering to the subject an effective amount of a TNFα inhibitor, such that the anemia is treated. The invention also pertains to methods wherein the TNFα inhibitor is administered in combination with another therapeutic agent to treat anemia. Various aspects of the invention relate to treatment with antibodies and antibody fragments, and pharmaceutical compositions comprising a TNFα inhibitor, and a pharmaceutically acceptable carrier for the treatment of anemia.

Definitions

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNFα" (abbreviated herein as hTNFα, or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNFα is described further in, for example, Pennica, D., *et al.* (1984) *Nature* 312:724-729; Davis, J.M., *et al.* (1987) *Biochemistry* 26:1322-1326; and Jones, E.Y., *et al.* (1989) *Nature* 338:225-228. The term human TNFα is intended to include recombinant human TNFα (rhTNFα), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, MN). TNFα is also referred to as TNF.

The term "TNFα inhibitor" includes agents which inhibit TNFα. Examples of TNFα inhibitors include etanercept (Enbrel®, Amgen), infliximab (Remicade®, Johnson and Johnson), human anti-TNF monoclonal antibody (D2E7/HUMIRA®, Abbott Laboratories), CDP 571 (Celltech), and CDP 870 (Celltech) and other compounds which inhibit TNFα activity, such that when administered to a subject suffering from or at risk of suffering from a disorder in which TNFα activity is detrimental, the disorder is treated. In one embodiment, a TNFα inhibitor is a compound, excluding etanercept and infliximab, which inhibits TNFα activity. In another embodiment, the TNFα inhibitors of the invention are used to treat a TNFα-related disorder, as described in more detail in section II. In one embodiment, the TNFα inhibitor, excluding etanercept and infliximab, is used to treat a TNFα-related disorder. In another embodiment, the TNFα inhibitor, excluding etanercept and infliximab, is used to treat anemia. The term also includes each of the anti-TNFα human antibodies and antibody portions described herein as well as those described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356.

The term "antibody," as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The antibodies of the invention are described in further detail in U.S. Patent Nos. 6,090,382; 6,258,562; and 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, hTNFα). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883) . Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123). The antibody portions of the invention are described in further detail in U.S. Patent Nos. 6,090,382, 6,258,562, 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

Binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies. Other than “bispecific” or “bifunctional” immunoglobulins or antibodies, an immunoglobulin or

antibody is understood to have each of its binding sites identical. A "bispecific" or "bifunctional antibody" is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny *et al.*, *J. Immunol.* 148, 1547-1553 (1992).

As used herein, the term "anemia" includes any disease, disorder, or condition characterized by, caused by, or related to any deficiency in the ability of blood to transport oxygen, deficiency in red blood cells, deficiency in hemoglobin, or deficiency in total blood volume. Anemia may be determined by comparing either hemoglobin (grams/deciliter), hematocrit (percentage of blood volume occupied by red blood cells) or red blood cell count (number of red blood cells times 10^6/microliter) with "normal" values. These normal values are arbitrarily set as the mean.+-.2 standard deviations of values in a healthy population. The normal ranges of blood parameters in adults are as follows: hemoglobin (gm/dl) 12.0-17.7; hematocrit (%) 36-52; Red blood cell count (number of red blood cells x 10^6/ul) 4.0-6.0; mean cell volume(fl) 80-100 (Adapted from Nathan, D.G. in Cecil Textbook of Medicine, (1992), J. B. Wyngaarden, L. H. Smith and J. C. Bennett, ed. W. B. Saunders Co., Philadelphia, pages 817-836, incorporated herein by reference). However, these normal ranges must be adjusted for persons living at altitude as well as for differences in race and gender.

Anemia may be masked by dehydration, where reduced plasma volume yields apparently normal hemoglobin concentrations, and likewise anemia can be mimicked by increased plasma volume, as in pregnancy. Thus the diagnosis of anemia can be made using published values as a guideline, but must be determined by a clinician skilled in the art.

Examples of "anemias" include, but are not limited to, anemias related to rheumatoid arthritis, anemias of infection and chronic inflammatory diseases, iron deficiency anemia, autoimmune hemolytic anemia, myelophthisic anemia, aplastic anemia, hypoplastic anemia, pure red cell aplasia and anemia associated with renal failure or endocrine disorders, megaloblastic anemias, defects in heme or globin synthesis, anemia caused by a structural defect in red blood cells, *e.g.,* sickle-cell anemia, and anemias of unknown origins such as sideroblastic anemia, anemia

associated with chronic infections such as malaria, trypanosomiasis, HIV, hepatitis virus or other viruses, and myelophthisic anemias caused by marrow deficiencies.

Anemias related to rheumatoid arthritis include, for example, anemia of chronic disease, iron deficiency anemia, and autoimmune hemolytic anemia. As used herein, the term "anemia of chronic disease" refers to an anemia which develops as a result of extended infection or inflamation. Certain chronic infections and inflammatory diseases cause several changes in the blood production (hematopoietic) system. These include a slightly shortened red blood cell life span and sequestration of iron in inflammatory cells called macrophages, resulting in a decrease in the amount of iron that is available to make red blood cells. In the presence of these effects a low to moderate grade anemia develops. The symptoms of the anemia may go unnoticed in the face of the primary disease.

Conditions associated with anemia of infection and chronic inflammatory diseases include such diverse diseases as chronic bacterial endocarditis, osteomyelitis, rheumatoid arthritis, juvenile rheumatoid arthritis, rheumatic fever, Crohn's disease, and ulcerative colitis.

As used herein the term "iron deficiency anemia" refers to a decrease in the number of red cells in the blood caused by too little iron. Iron deficiency anemia is the most common form of anemia. Approximately 20% of women, 50% of pregnant women, and 3% of men are iron deficient. The causes of iron deficiency are too little iron in the diet, and poor absorption of iron by the body. It can also occur secondary to bleeding in patients with rheumatoid arthritis.

As used herein the term "autoimmune hemolytic anemia" refers to a disorder in which the red blood cells are destroyed faster than the bone marrow can produce them. Red blood cells are produced healthy but are later destroyed by becoming trapped in the spleen, destroyed by infection, or destroyed from drugs that can affect red blood cells. Autoimmune hemolytic anemia is also referred to as extrinsic hemolytic anemia. Some of the causes of autoimmune hemolytic anemia are, for example, autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematous (SLE, or lupus), Wiskott-Aldrich syndrome, or ulcerative colitis; infections, such as hepatitis, cytomegalovirus (CMV), Epstein-Barr virus (EBV), typhoid fever, E. coli, or streptococcus; medications, such as penicillin, antimalaria medications, sulfa medications, or acetaminophen; or leukemia or lymphoma. The most common symptoms of autoimmune hemolytic anemia

are, for example, abnormal paleness or lack of color of the skin; jaundice or yellowing the skin, eyes, and mouth; dark color to urine; fever; weakness; dizziness; enlargement of the spleen and liver; and increased heart rate (tachycardia). Treatment may include, for example, vitamin and mineral supplements; change in diet; medication; treatment of the causative disease; and splenectomy (surgery to remove the spleen).

A "conservative amino acid substitution," as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

The term "human antibody," as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody," as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline

immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

An "isolated antibody," as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNFα is substantially free of antibodies that specifically bind antigens other than hTNFα). An isolated antibody that specifically binds hTNFα may, however, have cross-reactivity to other antigens, such as hTNFα molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

A "neutralizing antibody," as used herein (or an "antibody that neutralized hTNFα activity"), is intended to refer to an antibody whose binding to hTNFα results in inhibition of the biological activity of hTNFα. This inhibition of the biological activity of hTNFα can be assessed by measuring one or more indicators of hTNFα biological activity, such as hTNFα-induced cytotoxicity (either *in vitro* or *in vivo*), hTNFα-induced cellular activation and hTNFα binding to hTNFα receptors. These indicators of hTNFα biological activity can be assessed by one or more of several standard *in vitro* or *in vivo* assays known in the art (see U.S. Patent No. 6,090,382). Preferably, the ability of an antibody to neutralize hTNFα activity is assessed by inhibition of hTNFα-induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNFα activity, the ability of an antibody to inhibit hTNFα-induced expression of ELAM-1 on HUVEC, as a measure of hTNFα-induced cellular activation, can be assessed.

The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Example 1 and Jönsson, U., *et al.* (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., *et al.* (1991) *Biotechniques* 11:620-627; Johnsson, B., *et al.*

(1995) *J. Mol. Recognit.* 8:125-131; and Johnnson, B., *et al.* (1991) *Anal. Biochem.* 198:268-277.

The term "K_{off}," as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term "K_d," as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term "IC_{50}," as used herein, is intended to refer to the concentration of the inhibitor required to inhibit the biological endpoint of interest, e.g., neutralize cytotoxicity activity.

The term "nucleic acid molecule," as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule," as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind hTNFα, is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNFα, which other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an anti-hTNFα antibody contains no other sequences encoding other VH regions that bind antigens other than hTNFα.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to

herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

The term "dosing," as used herein, refers to the administration of a substance (*e.g.*, an anti-TNFα antibody) to achieve a therapeutic objective (*e.g.*, the treatment of a TNFα-associated disorder).

The terms "biweekly dosing regimen," "biweekly dosing," and "biweekly administration," as used herein, refer to the time course of administering a substance (*e.g.*, an anti-TNFα antibody) to a subject to achieve a therapeutic objective (*e.g.*, the treatment of a TNFα-associated disorder). The biweekly dosing regimen is not intended to include a weekly dosing regimen. Preferably, the substance is administered every 9-19 days, more preferably, every 11-17 days, even more preferably, every 13-15 days, and most preferably, every 14 days.

The term "combination" as in the phrase "a first agent in combination with a second agent" includes co-administration of a first agent and a second agent, which for example may be dissolved or intermixed in the same pharmaceutically acceptable carrier, or administration of a first agent, followed by the second agent, or administration of the second agent, followed by the first agent. The present invention, therefore, includes methods of combination therapeutic treatment and combination pharmaceutical compositions.

The term "concomitant" as in the phrase "concomitant therapeutic treatment" includes administering an agent in the presence of a second agent. A concomitant therapeutic treatment method includes methods in which the first, second, third, or additional agents are co-administered. A concomitant therapeutic treatment method also includes methods in which the first or additional agents are administered in the presence of a second or additional agents, wherein the second or additional agents, for example, may have been previously administered. A concomitant therapeutic treatment method may be executed step-wise by different actors. For example, one actor may administer to a subject a first agent and a second actor may to administer to the subject a second agent, and the administering steps may be executed at the same time, or nearly the same time, or at distant times, so long as the first agent (and additional agents) are after administration in the presence of the second agent (and additional agents). The actor and the subject may be the same entity (*e.g.*, human).

The term "combination therapy," as used herein, refers to the administration of two or more therapeutic substances, *e.g.*, an anti-TNFα antibody and another drug, such as a DMARD or NSAID. The other drug(s) may be administered concomitant with, prior to, or following the administration of an anti-TNFα antibody.

The term "kit" as used herein refers to a packaged product comprising components with which to administer the TNFα antibody of the invention for treatment of a TNFα-related disorder. The kit preferably comprises a box or container that holds the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which are preferably contained within plastic, polyethylene, polypropylene, ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can also include instructions for administering the TNFα antibody of the invention.

Various aspects of the invention are described in further detail herein.

I. TNFα Inhibitors of the Invention

This invention provides methods of treating anemia in which the administration of a TNFα inhibitor is beneficial. In one embodiment, these methods includes administration of isolated human antibodies, or antigen-binding portions thereof, that bind to human TNFα with high affinity, a low off rate and high neutralizing capacity.

Preferably, the human antibodies of the invention are recombinant, neutralizing human anti-hTNFα antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 (the amino acid sequence of the D2E7 VL region is shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is shown in SEQ ID NO: 2). D2E7 is also referred to as HUMIRA® and adalimumab. The properties of D2E7 have been described in Salfeld *et al.*, U.S. patent No. 6,090,382, which is incorporated by reference herein.

In one embodiment, the treatment of the invention includes the administration of D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNFα with low dissociation kinetics and high neutralizing capacity. In one embodiment, the invention provides treatment with an isolated human antibody, or an antigen-binding portion thereof, that dissociates from human TNFα with a K_d of 1×10^{-8} M or less and a K_{off} rate constant of 1×10^{-3} s^{-1} or less, both determined by surface plasmon resonance, and neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-7} M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less, or even more preferably, with a K_{off} of 1×10^{-4} s^{-1} or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNFα cytotoxicity in a standard *in vitro* L929 assay with an IC_{50} of 1×10^{-8} M or less, even more preferably with an IC_{50} of 1×10^{-9} M or less and still more preferably with an IC_{50} of 1×10^{-10} M or less. In a preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof.

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in another aspect, the invention pertains to methods of treating disorders in which the TNFα activity is detrimental by administering human antibodies that have slow dissociation kinetics for association with hTNFα and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7.

Position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VL CDR3 comprises the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the K_{off}. Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the D2E7 heavy and light chains is amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the K_{off}. Still further, the skilled artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More preferably, no more than one to three conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNFα. Positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3 appear to be critical for interaction with hTNFα and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above) (*see* U.S. Patent No. 6,090,382).

Accordingly, in another embodiment, the invention provides methods of treating anemia by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains the following characteristics:

a) dissociates from human TNFα with a K_{off} rate constant of 1×10^{-3} s^{-1} or less, as determined by surface plasmon resonance;

b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1,

4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;

c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 5×10^{-4} s^{-1} or less. Even more preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNFα with a K_{off} of 1×10^{-4} s^{-1} or less.

In yet another embodiment, the invention provides methods of treating anemia by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1). The framework regions for VL preferably are from the V_KI human germline family, more preferably from the A20 human germline Vk gene and most preferably from the D2E7 VL framework sequences shown in Figures 1A and 1B of U.S. Patent No. 6,090,382. The framework regions for VH preferably are from the V_H3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in Figures 2A and 2B U.S. Patent No. 6,090,382.

Accordingly, in another embodiment, the invention provides methods of treating anemia by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides methods of treating anemia in which the administration of an anti-TNFα antibody is beneficial administration of an isolated human antibody, or an antigen-binding portions thereof. The antibody or antigen-binding portion thereof preferably contains D2E7-related VL and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In another embodiment, the TNFα inhibitor of the invention is etanercept (described in WO 91/03553 and WO 09/406476), infliximab (described in U.S. Patent No. 5,656,272), CDP571 (a humanized monoclonal anti-TNF-alpha IgG4 antibody), CDP 870 (a humanized monoclonal anti-TNF-alpha antibody fragment),

D2E7/HUMIRA® (a human anti-TNF mAb), soluble TNF receptor Type I, or a pegylated soluble TNF receptor Type I (PEGs TNF-R1).

The TNFα antibody of the invention can be modified. In some embodiments, the TNFα antibody or antigen binding fragments thereof, is chemically modified to provide a desired effect. For example, pegylation of antibodies and antibody fragments of the invention may be carried out by any of the pegylation reactions known in the art, as described, for example, in the following references: *Focus on Growth Factors* 3:4-10 (1992); EP 0 154 316; and EP 0 401 384 (each of which is incorporated by reference herein in its entirety). Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). A preferred water-soluble polymer for pegylation of the antibodies and antibody fragments of the invention is polyethylene glycol (PEG). As used herein, "polyethylene glycol" is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-ClO) alkoxy- or aryloxy-polyethylene glycol.

Methods for preparing pegylated antibodies and antibody fragments of the invention will generally comprise the steps of (a) reacting the antibody or antibody fragment with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby the antibody or antibody fragment becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

Pegylated antibodies and antibody fragments may generally be used to treat spodyloarthropathies by administration of the TNFα antibodies and antibody fragments described herein. Generally the pegylated antibodies and antibody fragments have increased half-life, as compared to the nonpegylated antibodies and antibody fragments. The pegylated antibodies and antibody fragments may be employed alone, together, or in combination with other pharmaceutical compositions.

In yet another embodiment of the invention, TNFα antibodies or fragments thereof can be altered wherein the constant region of the antibody is modified to reduce at least one constant region-mediated biological effector function relative to an unmodified antibody. To modify an antibody of the invention such that it exhibits

reduced binding to the Fc receptor, the immunoglobulin constant region segment of the antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see *e.g.*, Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. *et al.* (1991) *J. of Immunol.* 147:2657-2662). Reduction in FcR binding ability of the antibody may also reduce other effector functions which rely on FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity.

An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (*e.g.*, another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and otherwise modified forms of the human anti-hTNFα antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate associate of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-napthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and

diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

An antibody, or antibody portion, of the invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning; A Laboratory Manual*, *Second Edition*, Cold Spring Harbor, N.Y., (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V_H Sequences Reveals about Fifty Groups of V_H Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V_{78} Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V_H3 family of human germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain

variable region of D2E7, or a D2E7-related antibody, a member of the V_KI family of human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-31 germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited *supra*, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA

fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid sequence $(Gly_4\text{-}Ser)_3$, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; McCafferty *et al.*, *Nature* (1990) 348:552-554).

To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to

insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication)

and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr$^-$ host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above

procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNFα. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNFα by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

Recombinant human antibodies of the invention in addition to D2E7 or an antigen binding portion thereof, or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating

and screening antibody display libraries can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.* PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; McCafferty *et al.*, *Nature* (1990) 348:552-554; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982. Methods of isolating human antibodies with high affinity and a low off rate constant for hTNFα are described in U.S. Patent Nos. 6,090,382, 6,258,562, and 6,509,015, each of which is incorporated by reference herein.

II. Uses of TNFα Inhibitors of the Invention

In an embodiment, the invention provides a method for inhibiting TNFα activity in a subject suffering from anemia in which TNFα activity is detrimental. In one embodiment, the TNFα inhibitor is D2E7, also referred to as HUMIRA® (adalimumab).

TNFα has been implicated in the pathophysiology of a wide variety of anemias (see *e.g.*, Jongen-Lavrencic M., *et al.* (1997) *J. Rheumatol.*24(8):1504-9; Demeter J., *et al.* (2002) *Ann Hematol.* 81(10):566-9; DiCato M., (2003) *The Oncologist* 8 (suppl 1):19-21). The invention provides methods for inhibiting TNFα activity in a subject suffering from such a disorder, which method comprises administering to the subject an antibody, antibody portion, or other TNFα inhibitor of the invention such that TNFα activity in the subject suffering from anemia is inhibited. Preferably, the TNFα is human TNFα and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNFα with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has been introduced hTNFα (*e.g.*, by administration of hTNFα or by expression of an hTNFα transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed

further below). Moreover, an antibody of the invention can be administered to a non-human mammal expressing a TNFα with which the antibody cross-reacts (*e.g.*, a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (*e.g.*, testing of dosages and time courses of administration). Examples of animal models for evaluating the efficacy of a TNFα antibody for the treatment of anemia include rats inoculated with peptidolglycan-polysaccharide polymers (see Coccia *et al.*, (2001) *Exp Hematology.* 29:1201-1209).

As used herein, the term "anemia disorder in which TNFα activity is detrimental" is intended to include diseases and other disorders in which the presence of TNFα in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the disorder. Accordingly, a disorder in which TNFα activity is detrimental is a disorder in which inhibition of TNFα activity is expected to alleviate the symptoms and/or progression of the disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNFα in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNFα in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an anti-TNFα antibody as described above. There are numerous examples of disorders in which TNFα activity is detrimental. An example of a disorder in which TNFα activity is detrimental is anemia. The use of the antibodies, antibody portions, and other TNFα inhibitors of the invention in the treatment of specific disorders, *e.g.*, anemias, are discussed further below. In certain embodiments, the antibody, antibody portion, or other TNFα inhibitor of the invention is administered to the subject in combination with another therapeutic agent, as described below.

The term “anemia” as used herein, refers to an abnormally low number of circulating red cells or a decreased concentration of hemoglobin in the blood.

In one embodiment, the invention features a method for treating an anemia disorder in which TNFα activity is detrimental, comprising administering to a subject an effective amount of a TNFα inhibitor, such that the disorder is treated, wherein said disorder is anemia. In one embodiment, the anemia is associated with rheumatoid

arthritis. Examples of anemia related to rheumatoid arthritis include, for example, anemia of chronic disease, iron deficiency anemia, and autoimmune hemolytic anemia.

In one embodiment, the invention provides a method of treating anemia. In another embodiment, the invention provides a method of treating anemias related to, for example, anemias related to rheumatoid arthritis, anemias of infection and chronic inflammatory diseases, iron deficiency anemia, autoimmune hemolytic anemia, myelophthisic anemia, aplastic anemia, hypoplastic anemia, pure red cell aplasia and anemia associated with renal failure or endocrine disorders, megaloblastic anemias, defects in heme or globin synthesis, anemia caused by a structural defect in red blood cells, *e.g.*, sickle-cell anemia, and anemias of unknown origins such as sideroblastic anemia, anemia associated with chronic infections such as malaria, trypanosomiasis, HIV, hepatitis virus or other viruses, and myelophthisic anemias caused by marrow deficiencies.

III. Pharmaceutical Compositions and Pharmaceutical Administration

A. *Compositions and Administration*

The antibodies, antibody-portions, and other TNFα inhibitors of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises an antibody, antibody portion, or other TNFα inhibitor of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody, antibody portion, or other TNFα inhibitor.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies or other TNFα inhibitors. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody or other TNFα inhibitor is administered by intravenous infusion or injection. In another preferred embodiment, the antibody or other TNFα inhibitor is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody, antibody portion, or other TNFα inhibitor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic

agents. For example, an anti- hTNFα antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more DMARD or one or more NSAID or one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751) or any combination thereof. Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

In one embodiment, the invention includes pharmaceutical compositions comprising an effective amount of a TNFα inhibitor and a pharmaceutically acceptable carrier, wherein the effective amount of the TNFα inhibitor may be effective to treat anemia, including anemias associated with rheumatoid arthritis, such as, for example, anemia of chronic disease, iron deficiency anemia, and autoimmune hemolytic anemia.

The antibodies, antibody-portions, and other TNFα inhibitors of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

The TNFα antibodies of the invention can also be administered in the form of protein crystal formulations which include a combination of protein crystals encapsulated

within a polymeric carrier to form coated particles. The coated particles of the protein crystal formulation may have a spherical morphology and be microspheres of up to 500 micro meters in diameter or they may have some other morphology and be microparticulates. The enhanced concentration of protein crystals allows the antibody of the invention to be delivered subcutaneously. In one embodiment, the TNFα antibodies of the invention are delivered via a protein delivery system, wherein one or more of a protein crystal formulation or composition, is administered to a subject with anemia.

In certain embodiments, an antibody, antibody portion, or other TNFα inhibitor of the invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody, antibody portion, or other TNFα inhibitor may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody, antibody portion, other TNFα inhibitor to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody, antibody portion, or other TNFα inhibitor are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 10-100 mg, more preferably 20-80 mg and most preferably about 40 mg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. Ranges intermediate to the above recited concentrations, *e.g.*, about 6-144 mg/ml, are also intended to be part of this invention. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian

subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody or antibody portion of the invention is 10-150 mg, more preferably 20-80 mg and most preferably about 40 mg. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition. Ranges intermediate to the above recited concentrations, *e.g.*, about 6-144 mg/ml, are also intended to be part of this invention. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The invention also pertains to packaged pharmaceutical compositions which comprise a TNFα inhibitor of the invention and instructions for using the inhibitor to treat anemia, as described above.

Another aspect of the invention pertains to kits containing a pharmaceutical composition comprising an anti-TNFα antibody and a pharmaceutically acceptable carrier and one or more pharmaceutical compositions each comprising a drug useful for treating anemia, and a pharmaceutically acceptable carrier.

Alternatively, the kit comprises a single pharmaceutical composition comprising an anti-TNFα antibody, one or more drugs useful for treating anemia, and a pharmaceutically acceptable carrier. The kits contain instructions for dosing of the pharmaceutical compositions for the treatment of a disorder in which the administration of an anti-TNFα antibody is beneficial, such as anemia.

The invention also pertains to packaged pharmaceutical compositions or kits which comprise a TNFα inhibitor of the invention and instructions for using the inhibitor to treat a particular disorder in which TNFα activity is detrimental, as described above. The package or kit alternatively can contain the TNFα inhibitor and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged pharmaceuticals or kits further can include a second agent (as described herein) packaged with or copromoted with instructions for using the second agent with a first agent (as described herein).

B. *Additional therapeutic agents*

The invention pertains to pharmaceutical compositions and methods of use thereof for the treatment of anemia. The pharmaceutical compositions comprise a first agent that prevents or inhibits anemia. The pharmaceutical composition also may comprise a second agent that is an active pharmaceutical ingredient; that is, the second agent is therapeutic and its function is beyond that of an inactive ingredient, such as a pharmaceutical carrier, preservative, diluent, or buffer. The second agent may be useful in treating or preventing anemia. The second agent may diminish or treat at least one symptom(s) associated with the targeted disease. The first and second agents may exert their biological effects by similar or unrelated mechanisms of action; or either one or both of the first and second agents may exert their biological effects by a multiplicity of mechanisms of action. A pharmaceutical composition may also comprise a third compound, or even more yet, wherein the third (and fourth, etc.) compound has the same characteristics of a second agent.

It should be understood that the pharmaceutical compositions described herein may have the first and second, third, or additional agents in the same pharmaceutically acceptable carrier or in a different pharmaceutically acceptable carrier for each described embodiment. It further should be understood that the first, second, third and additional agent may be administered simultaneously or sequentially within described embodiments. Alternatively, a first and second agent may be administered simultaneously, and a third or additional agent may be administered before or after the first two agents.

The combination of agents used within the methods and pharmaceutical compositions described herein may have a therapeutic additive or synergistic effect on the condition(s) or disease(s) targeted for treatment. The combination of agents used within the methods or pharmaceutical compositions described herein also may reduce a detrimental effect associated with at least one of the agents when administered alone or without the other agent(s) of the particular pharmaceutical composition. For example, the toxicity of side effects of one agent may be attenuated by another agent of the composition, thus allowing a higher dosage, improving patient compliance, and improving therapeutic outcome. The additive or synergistic effects, benefits, and advantages of the compositions apply to classes of therapeutic agents, either structural or functional classes, or to individual compounds themselves.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents that are useful for treating disorders in which TNFα activity is detrimental. For example, an anti-hTNFα antibody, antibody portion, or other TNFα inhibitor of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNFα receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNFα production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies or other TNFα inhibitors of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies. Specific therapeutic agent(s) are generally selected based on the particular disorder being treated, as discussed below.

Nonlimiting examples of therapeutic agents with which an antibody, antibody portion, or other TNFα inhibitor of the invention can be combined include the following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNFα antibody; Celltech/Bayer); cA2/infliximab (chimeric anti-TNFα antibody; Centocor); 75

kdTNFR-IgG/etanercept (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNF-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see *e.g.*, *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see *e.g.*, *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2Rα; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNF(soluble TNF binding protein; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol. 268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate; thalidomide (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (*e.g.*, Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284); T-614 (cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7, pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1β converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70

or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitors of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase inhibitors; anti-IL-12 antibodies; anti-IL-18 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil; cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see *e.g.*, DeLuca et al. (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amiprilose (therafectin); cladribine (2-chlorodeoxyadenosine); azaribine; methotrexate; antivirals; and immune modulating agents. Any of the above-mentioned agents can be administered in combination with the TNFα antibody of the invention to treat anemia. In one embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from rheumatoid arthritis in addition to anemia.

In one embodiment, the TNFα antibody of the invention is administered in combination with one of the following agents for the treatment of rheumatoid arthritis: methotrexate, prednisone, celecoxib, folic acid, hydroxychloroquine sulfate, rofecoxib, etanercept, infliximab, leflunomide, naproxen, valdecoxib, sulfasalazine, methylprednisolone, ibuprofen, meloxicam, methylprednisolone acetate, gold sodium thiomalate, aspirin, azathioprine, triamcinolone acetonide, propxyphene napsylate/apap, folate, nabumetone, diclofenac, piroxicam, etodolac, diclofenac sodium, oxaprozin, oxycodone hcl, hydrocodone bitartrate/apap, diclofenac sodium/misoprostol, fentanyl, anakinra, human recombinant, tramadol hcl, salsalate, sulindac,

cyanocobalamin/fa/pyridoxine, acetaminophen, alendronate sodium, prednisolone, morphine sulfate, lidocaine hydrochloride, indomethacin, glucosamine sulfate/chondroitin, cyclosporine, amitriptyline hcl, sulfadiazine, oxycodone hcl/acetaminophen, olopatadine hcl, misoprostol, naproxen sodium, omeprazole, mycophenolate mofetil, cyclophosphamide, rituximab, IL-1 TRAP, MRA, CTLA4-IG, IL-18 BP, ABT-874, ABT-325 (anti-IL 18), anti-IL 15, BIRB-796, SCIO-469, VX-702, AMG-548, VX-740, Roflumilast, IC-485, CDC-801, and mesopram. In another embodiment, the TNFα antibody of the invention is administered for the treatment of a anemia in combination with one of the above mentioned agents for the treatment of rheumatoid arthritis.

In one embodiment, the TNFα antibody of the invention is administered in combination with one of the following agents for the treatment of anemia in which TNFα activity is detrimental: anti-IL12 antibody (ABT 874); anti-IL18 antibody (ABT 325); small molecule inhibitor of LCK; small molecule inhibitor of COT; anti-IL1 antibody; small molecule inhibitor of MK2; anti-CD19 antibody; small molecule inhibitor of CXCR3; small molecule inhibitor of CCR5; small molecule inhibitor of CCR11 anti-E/L selectin antibody; small molecule inhibitor of P2X7; small molecule inhibitor of IRAK-4; small molecule agonist of glucocorticoid receptor; anti-C5a receptor antibody; small molecule inhibitor of C5a receptor; anti-CD32 antibody; Erythropoetin; iron; and CD32 as a therapeutic protein.

In yet another embodiment, the TNFα antibody of the invention is administered in combination with an antibiotic or anti-infective agent. Anti-infective agents include those agents known in the art to treat viral, fungal, parasitic or bacterial infections. The term, "antibiotic," as used herein, refers to a chemical substance that inhibits the growth of, or kills, microorganisms. Encompassed by this term are antibioteic produced by a microorganism, as well as synthetic antibiotics (e.g., analogs) known in the art. Antibiotics include, but are not limited to, clarithromycin (Biaxin®), ciprofloxacin (Cipro®), and metronidazole (Flagyl®).

Any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from a disorder in which TNFα is detrimental, *e.g.,* anemia, in combination with the TNFα antibody of the invention. In one embodiment, any one of the above-mentioned therapeutic agents, alone or in

combination therewith, can be administered to a subject suffering from rheumatoid arthritis in addition to a TNFα antibody to treat anemia. In another embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered in combination with the TNFα antibody of the invention, to a subject suffering from anemia.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Example 1: TNFα Inhibitor in Iron Deficiency Anemia

Study of D2E7 in rat model of iron deficiency

The following study is performed using the rat animal model of iron deficiency anemia (Catani et al (2003) *Braz. J. Med. Biol. Res.* 36;693). Male Wistar-EPM rats (approximately three weeks old) are fed an AIN-93G (American Institute of Nutrition Rodent Diets) iron-free diet for a period of two weeks to induce iron deficiency anemia. Rats are administered doses of D2E7 or a placebo. Blood samples are taken pre and post treatment to determine hemoglobin and hematocrit values. For the analysis of hematocrit and hemoglobin concentration, blood is collected and mixed with 5ml of 0.5 M EDTA. Hematocrit is determined by centrifugation of blood in sealed heparinized capillaries. Hemoglobin concentrations are calculated from the absorbance of cyanmethemoglobin at 546 nm. Rats are examined to determine if there was an improved hematocrit measurement.

Example 2: TNFα inhibitor Study of Chronic Disease Anemia

Study of D2E7 on anemia associated with chronic inflammatory disease

The following study is performed using a rat model of anemia of chronic disease (Coccia et al, (2001) *Exp. Hematology* 29;1201). Eight to ten week old female Lewis

rats are inoculated on day 0 with an intraperitoneal (i.p.) injection of peptidoglycan-polysaccharide polymers (PG-PSP) suspended in 0.85% saline equilibrated to a dose of 15 μg rhamnose/kg. Blood is collected via tail veins into EDTA-coated Microtainer tubes and complete blood counts (CBC) are performed on an ADVA 120 Hematology System calibrated for rat blood. An additional blood sample is collected and separated on Microtainer serum separator centrifuge tubes and sera are analyzed for iron, bilirubin, and endogenous EPO concentrations. Rats are administered doses of D2E7 or a placebo, and examined for improved iron, bilirubin, and EPO concentration measurements.

Example 3: TNFα Inhibitor on Anemia

Study of D2E7 in human subjects with anemia

Patients who exhibit symptoms commonly associated with anemia are examined and tested to determine if they suffer from anemia. Symptoms commonly associated with anemia are fatigue, chest pain, shortness of breath, pale complexion, and rapid heart rate. Examples of tests which indicate anemia are the complete blood count (CBC), reticulocyte count, and measurements of iron supply, including the serum iron, total iron-binding capacity, and serum ferritin. In patients with sever anemia and abnormalities in red blood cell morphology, a bone marrow aspirate and biopsy are important diagnostic tools. Patients who suffer from anemia are selected for the study.

In the CBC test, automated cell counters measure a number of parameters as part of the CBC, including the hemoglobin, red blood cell count, red blood cell volume distribution, platelet count, and white blood cell count. The counter also calculates the hematocrit (based on the RBC count and volume), the mean cell volume (MCV) (based on volume distribution), mean cell hemoglobin (MCH)(hemoglobin divided by hematocrit), and the red cell distribution width (RDW). The red cell indices and RDW are used together with a direct inspection of the Wright-stained blood smear to evaluate red blood cell morphology.

Like the CBC test, an accurate measure of the reticulocyte count is key to the initial classification of any anemia. Reticulocytes are newborn red blood cells that contain sufficient residual RNA that they can be stained with a supravital dye and counted as a percent of the circulating red cell population. In the basal state, the normal

reticulocyte count ranges from 1 to 2 percent according to the counting method. This correlates with the normal dialy replacement of approximately 1 percent of the circulating red blood cell population. Increases in the reticulocyte count provide a reliable measure of the red blood cell production response to anemia.

To use the reticulocyte count as a production measure, it must first be corrected for changes in the patient's hematocrit and for the effect of erythropoietin on the early release of marrow reticulocytes into circulation. The hematocrit (HCT) correction converts the reicultocyte percentage to an absolute number:

$$\%\ \text{Reticulocytes} \times \frac{\text{patient HCT}}{45\%} = \text{absolute \% reticulocytes}$$

The marrow reticulocyte ("shift") correction involves dividing the absolute percentage by a factor of 1.5 to 2.5 whenever there is prominent polychromasia on the peripheral blood smear. The shift correction should always be applied to any patient with anemia and a very high reticulocyte count to provide a true index of effective red blood cell production. A normal patient will respond to a hematocrit less than 30 percent with a two-to three-fold increase in the reticulocyte production index. This measure alone, therefore, will confirm the fact that the patient has an appropriate erythropoietin response, a normal erythroid marrow, and sufficient iron supply to meet the challenge. When the reticulocyte index falls below 2, a defect in marrow proliferation or precursor maturation must be present.

Standard measures of iron supply include the serum iron, transferring iron-binding capacity (TIBC), and the serum ferritin level. The normal serum iron ranges from 9 to 27 μmol/L (50 to 150 μg/dL), while the normal TIBC is 54 to 64 μmol/L (300 to 360 μg/dL). Therefore, in the basal state, only 30 to 50 percent of the transferring in circulation is saturated with iron. Important information is provided by each measurement as well as the calculated percent saturation. The serum ferritin is used to evaluate body iron stores. Adult males have serum ferritin levels of between 50 and 150 mg/L, corresponding to iron stores of from 600 to 1000 mg. Adult females have lower serum ferritin levels (15 to 50 mg/L) and smaller iron stores (0 to 300 mg). Lower serum ferritin levels are observed as iron stores are depleted; levels below 15mg/L indicate store exhaustion and iron deficiency.

A sample of bone marrow is readily obtained by needle aspirate or biopsy. It is of greatest value in patients who have a hypoproliferative anemia or a disorder of red

blood cell maturation, providing valuable information as to marrow structure and cellularity, as well as precursor proliferation and maturation. The ratio of erythroid to granulocytic precursors (E/G ration) is used to asses the proliferative capacity of erythorid precursors. A patient with hypoproliferative anemia and a reticulocyte index <2 will demonstrate an E/G ratio ≤ 1:3 or 1:2. In contrast, the hemolytic anemia patient with a production index ≥ 3 to 5 will have an E/G ratio >1:1. Red cell precursor maturation defects are identified from the mismatch between the E/G ratio and reticulocyte production index. These individuals demonstrate and E/G ratio of greater than 1:1 together with a low reticulocyte index, typical of the ineffective erythorpoiesis of a maturation disorder.

Following baseline measurements, patients begin receiving treatment. They are randomized and treated with either D2E7 or placebo in a blinded fashion. Patient's complete blood count (CBC), reticulocyte count, and measurements of iron supply are monitored at least every two weeks.

Example 4: Crystallization of D2E7 F(ab)'$_2$ fragment

Generation and purification of the D2E7 F(ab)'$_2$ Fragment

A D2E7 F(ab)'$_2$ fragment was generated and purified according to the following procedure. Two ml of D2E7 IgG (approximately 63 mg/ml) was dialyzed against 1 liter of Buffer A (20 mM NaOAc, pH 4) overnight. After dialysis, the protein was diluted to a concentration of 20 mg/ml. Immobilized pepsin (Pierce; 6.7 ml of slurry) was mixed with 27 ml of Buffer A, mixed, and centrifuged (Beckman floor centrifuge, 5000 rpm, 10 min). The supernatant was removed, and this washing procedure was repeated twice more. The washed immobilized pepsin was re-suspended in 13.3 ml of Buffer A. D2E7 (7.275 ml, 20 mg/ml, 145.5 mg) was mixed with 7.725 ml of Buffer A Bnd 7.5 ml of the washed immobilized pepsin slurry. The D2E7/pepsin mixture was incubated at 37 °C for 4.5 hr with shaking (300 rpm). The immobilized pepsin was then separated by centrifugation. Analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was essentially complete (~115 kDa band unreduced, ~30 and ~32 kDa bands reduced).

The D2E7 F(ab)'$_2$ fragment was separated from intact D2E7 and Fc fragments using Protein A chromatography. One-half of the above reaction supernatant (10 ml)

was diluted with 10 ml of Buffer B (20 mM Na phosphate, pH 7), filtered through a 0.45 μm Acrodisk filter, and loaded onto a 5 ml Protein A Sepharose column (Pharmacia Hi-Trap; previously washed with 50 ml of Buffer B). Fractions were collected. After the protein mixture was loaded, the column was washed with Buffer B until the absorbance at 280 nm re-established a baseline. Bound proteins were eluted with 5 ml of Buffer C (100 mM citric acid, pH 3); these fractions were neutralized by adding 0.2 ml of 2 M Tris•HCl, pH 8.9. Fractions were analyzed by SDS-PAGE; those that contained the D2E7 $F(ab)'_2$ fragment were pooled (~42 ml). Protein concentrations were determined by absorbance at 280 nm in 6 M guanidine•HCl, pH 7 (calculated extinction coefficients: D2E7, 1.39 (AU-ml)/mg; $F(ab)'_2$, 1.36 (AU-ml)/mg). The flow-though pool contained ~38.2 mg protein (concentration, 0.91 mg/ml), which represents a 79% yield of $F(ab)'_2$ (theoretical yield is ~2/3 of starting material, divided by two [only half purified], i.e. ~48.5 mg).

The D2E7 $F(ab)'_2$ fragment was further purified by size-exclusion chromatography. The pooled Protein A flow-through was concentrated from ~42 to ~20 ml, and a portion (5 ml, ~7.5 mg) was then chromatographed on a Superdex 200 column (26/60, Pharmacia) previously equilibrated (and eluted) with Buffer D (20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA). Two peaks were noted by absorbance at 280 nm: Peak 1, eluting at 172–200 ml, consisted of $F(ab)'_2$ (analysis by SDS-PAGE; ~115 kDa band unreduced, ~30 and ~32 kDa bands reduced); Peak 2, eluting at 236–248 ml, consisted of low molecular weight fragment(s) (~15 kDa, reduced or unreduced). Peak 1 was concentrated to 5.3 mg/ml for crystallization trials.

Crystallization of the D2E7 $F(ab)'_2$ Fragment

The D2E7 $F(ab)'_2$ fragment (5.3 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method by mixing equal volumes of $F(ab)'_2$ and crystallization buffer (approx. 1 μl of each) and allowing the mixture to equilibrate against the crystallization Buffer Bt 4 or 18 °C. The crystallization buffers used consisted of the Hampton Research Crystal Screens I (solutions 1–48) and II (solutions 1–48), Emerald Biostructures Wizard Screens I and II (each solutions 1–48), and the Jena Biosciences screens 1–10 (each solutions 1–24). Crystals were obtained under many different conditions, as summarized in Table 1.

Table 1. Summary of crystallization conditions for the D2E7 F(ab)'$_2$ fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	32	4	2.0 M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 1	46	4	0.2 M $Ca(Oac)_2$, 0.1 M Na cacodylate pH 6.5, 18% PEG 8K	medium sized needle clusters
Hampton 1	48	4	0.1 M Tris HCl pH 8.5, 2.0 M $NH_4H_2PO_4$	micro needle clusters
Hampton 2	2	4	0.01 M hexadecyltrimethylammonium bromide, 0.5 M NaCl, 0.01 M $MgCl_2$	small shard crystals
Hampton 2	13	4	0.2 M $(NH_4)_2SO_4$, 0.1 M NaOAc pH 4.6, 30% PEG MME 2000	small needle clusters
Hampton 2	15	4	0.5 M $(NH_4)_2SO_4$, 0.1M NaOAc pH 5.6, 1.0M Li_2SO_4	large needle clusters
Hampton 2	16	4	0.5M NaCl, 0.1M NaOAc pH 5.6, 4% Ethylene Imine polymer	large irregular crystal
Hampton 1	34	18	0.1 NaOAc pH 4.6, 2.0 M Na Formate	needle clusters
Hampton 1	35	18	0.1M Hepes pH 7.5, 0.8M mono-sodium dihydrogen phosphate, 0.8M mono-potasium dihydrogen phosphate	needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2.0M NaCl	dense needle clusters
Hampton 2	12	18	0.1M $CdCl_2$, 0.1M NaOAc pH 4.6, 30% PEG 400	needles & amorphous crystals
Hampton 2	15	18	0.5M $(NH_4)_2SO_4$, 0.1M NaOAc pH 5.6, 1.0M Li_2SO_4	tiny needle clusters
Wizard I	27	4	1.2M NaH2PO4, 0.8M K2HPO4, 0.1M CAPS pH 10.5, 0.2 M Li_2SO_4	Medium large needle clusters
Wizard I	30	4	1.26M $(NH_4)_2SO_4$, 0.1 M NaOAc pH 4.5, 0.2M NaCl	small needle clusters
Wizard II	8	4	10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl	Large plate crystals grown in clusters
Wizard II	43	4	10% PEK 8K, 0.1M Tris pH 7.0, 0.2 M MgCl2	micro needle clusters
Wizard I	4	18	35% MPD, 0.1M Imidazole pH 8.0, 0.2M MgCl2	rod shaped crystal
Wizard I	27	18	1.2M NaH2PO4, 0.8M K2HPO4, 0.1M CAPS pH 10.5, 0.2 M Li_2SO_4	Needle clusters
Wizard II	7	18	30% PEG 3K, 0.1M Tris pH 8.5, 0.2M NaCl	tiny needle clusters
Wizard II	11	18	10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)2	tiny hexagonal or rhombohedral crystals
Wizard II	46	18	1.0M AP, 0.1M Imidazole pH 8.0, 0.2M NaCl	1 irregular crystal
JB 1	D6	4	30% PEG 3K, 0.1M Tris HCl pH 8.5, 0.2M Li_2SO_4	tiny needles in precipitate
JB 2	B6	4	20% PEG 4K, 0.1M Tris HCl pH 8.5, 0.2M Na Cacodylate	tiny needle cluster balls
JB 3	A1	4	8% PEG 4K, 0.8M LiCl, 0.1M Tris HCl pH 8.5	Large frost-like crystals
JB 3	B1	4	15% PEG 4K, 0.2M $(NH_4)_2SO_4$	tiny needle clusters
JB 3	D5	4	30% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH_4OAc	tiny needles in precipitate.
JB 4	B1	4	15% PEG 6K, 0.05M KCl, 0.01M $MgCl_2$	needle cluster balls
JB 3	A6	18	12% PEG 4K, 0.1M NaOAc pH 4.6, 0.2M NH_4OAc	needle clusters
JB 3	B1	18	15% PEG 4K, 0.2M $(NH_4)_2SO_4$	needle clusters in precipitate
JB 3	C6	18	25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH_4OAc	long, thin needles
JB 4	C5	18	8% PEG 8K, 0.2 M LiCl, 0.05M $MgSO_4$	frost-like crystals

Screen	Solution	Temp °C	Condition	Result
JB 5	A3	4	15% PEG 8K, 0.2M $(NH_4)_2SO_4$	long single needles in phase separation
JB 5	A4	4	15% PEG 8K, 0.5M Li_2SO_4	tiny needle clusters
JB 5	A5	4	15% PEG 8K, 0.1M Na MES pH 6.5, 0.2M $Ca(OAc)_2$	needle cluster balls
JB 6	B2	4	1.6M $(NH_4)_2SO_4$, 0.5 LiCl	tiny needle cluster balls
JB 6	C2	4	2.0 M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6	micro needle clusters
JB 10	D3	18	2.0M Na Formate, 0.1M NaOAc pH 4.6	needle clusters

Example 5: Crystallization of D2E7 Fab fragment

Generation and purification of the D2E7 Fab Fragment

A D2E7 Fab fragment was generated and purified according to the following procedure. Four ml of D2E7 IgG (diluted to about 20 mg/ml) was diluted with 4 ml of Buffer E (20 mM Na phosphate, 5 mM cysteine•HCl, 10 mM EDTA, pH7) and mixed with 6.5 ml of a slurry of immobilized papain (Pierce, 1%; previously washed twice with 26 ml of Buffer E). The D2E7/papain mixture was incubated at 37 °C overnight with shaking (300 rpm). The immobilized papain and precipitated protein were separated by centrifugation; analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was partially complete (~55, 50, 34, and 30 kDa bands unreduced, with some intact and partially digested D2E7 at ~115 and ~150 kDa; ~30 and ~32 kDa bands reduced, as well as a ~50 kDa band). Nonetheless, the digestion was halted and subjected to purification.

The D2E7 Fab fragment was purified by Protein A chromatography and Superdex 200 size-exclusion chromatography essentially as described above for the $F(ab)'_2$ fragment. The Protein A column flow-through pool (21 ml) contained ~9.2 mg (0.44 mg/ml), whereas the Protein A eluate (4 ml) contained ~19.5 mg (4.9 mg/ml). Analysis by SDS-PAGE indicated that the flow-through was essentially pure Fab fragment (~48 and ~30 kDa unreduced, broad band at ~30 kDa reduced), whereas the eluate was intact and partially-digested D2E7. The Fab fragment was further purified on a Superdex 200 column, eluting at 216–232 ml, i.e., as expected, after the $F(ab)'_2$

fragment but before the small Fc fragments. The D2E7 Fab fragment concentrated to 12.7 mg/ml for crystallization trials, as described below.

Crystallization of the D2E7 Fab Fragment

The D2E7 Fab fragment (12.7 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method essentially as described above for the $F(ab)'_2$ fragment. Crystals were obtained under many different conditions, as summarized in **Table 2**.

Table 2. Summary of crystallization conditions for the D2E7 Fab fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	4	4	0.1M Tris pH 8.5, 2M $(NH_4)_2SO_4$	wispy needles
Hampton 1	10	4	0.2M NH_4OAc, 0.1M NaOAc pH 4.6, 30% PEG 4K	wispy needle clusters
Hampton 1	18	4	0.2M $Mg(OAc)_2$, 0.1M Na Cacodylate pH 6.5, 20% PEG 8K	needle clusters
Hampton 1	20	4	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG 4K	tiny needle clusters
Hampton 1	32	4	2M $(NH_4)_2SO_4$	long, wispy needles
Hampton 1	33	4	4M Na Formate	tiny needle clusters
Hampton 1	38	4	0.1M Hepes pH 7.5	tiny needle clusters
Hampton 1	43	4	30% PEG 1500	tiny needle clusters
Hampton 1	46	4	0.2M $Ca(OAc)_2$, 0.1M Na Cacodylate pH 6.5, 18% PEG 8K	large plate clusters
Hampton 1	47	4	0.1M NaOAc pH 4.6, 2M $(NH_4)_2SO_4$	long, wispy needles
Hampton 2	1	4	2M NaCl, 10% PEG 6K	small plate clusters
Hampton 2	2	4	0.01M Hexadecyltrimethylammonium bromide, 0.5M NaCl, 0.01 $MgCl_2$	round & irregular plates
Hampton 2	5	4	2M $(NH_4)_2SO_4$, 5% isopropanol	long fiber ropes
Hampton 2	13	4	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny, wispy needle clusters
Hampton 2	14	4	0.2M K/Na Tatrate, 0.1M Na Citrate pH 5.6, 2M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 2	27	4	0.01M $ZnSO_4$, 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Hampton 2	28	4	30% MPD	tiny needle clusters
Hampton 1	4	18	0.1M Tris pH 8.5, 2M $(NH_4)_2SO_4$	needle clusters
Hampton 1	9	18	0.2M NH_4OAc, 0.1M Na Citrate pH 5.6, 30% PEG 4K	needle clusters
Hampton 1	17	18	0.2M Li_2SO_4, 0.1M Tris pH 8.5, 30% PEG 4K	long, wispy needles
Hampton 1	32	18	2M $(NH_4)_2SO_4$	needle clusters
Hampton 1	33	18	4M Na Formate	tiny needle clusters
Hampton 1	38	18	0.1M Hepes pH 7.5	fiber bundles
Hampton 1	43	18	30% PEG 1500	tiny needle clusters
Hampton 1	47	18	0.1M NaOAc pH 4.6, 2M $(NH_4)_2SO_4$	tiny needle clusters
Hampton 2	1	18	2M NaCl, 10% PEG 6K	long, wispy needle clusters
Hampton 2	5	18	2M $(NH_4)_2SO_4$, 5% 2-propanol	tiny needle clusters

Screen	Solution	Temp °C	Condition	Result
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2M NaCl	long, wispy needles
Hampton 2	13	18	0.2M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny needle clusters
Hampton 2	14	18	0.2M K/Na Tartrate, 0.1M Na Citrate pH 5.6, 2M $(NH_4)_2SO_4$	long wispy needles
Hampton 2	27	18	0.01M $ZnSO_4$, 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Wizard I	20	4	0.4M NaH_2PO_4/1.6M K_2HPO_4, 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	28	4	20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl	large orthorhombic plate clusters
Wizard I	31	4	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	wispy needle clusters
Wizard I	39	4	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li_2SO_4	needle clusters
Wizard II	3	4	20% PEG 8K, 0.1M Tris pH 8.5, 0.2M $MgCl_2$	large hexagonal or orthorhombic plate cluster in phase sep
Wizard II	4	4	2M $(NH_4)_2SO_4$, 0.1M Cacodylate pH 6.5, 0.2 NaCl	tiny needle clusters
Wizard II	9	4	2M $(NH_4)_2SO_4$, 0.1M phosphate citrate pH 4.2	tiny, wispy needle clusters
Wizard II	28	4	20% PEG 8K, 0.1M MES pH 6, 0.2M $Ca(OAc)_2$	tiny needle clusters; large wispy needle clusters
Wizard II	35	4	0.8M NaH_2PO_4/1.2M K_2HPO_4, 0.1M NaOAc pH 4.5	tiny fiber bundles
Wizard II	38	4	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li_2SO_4	long wispy needles
Wizard II	47	4	2.5M NaCl, 0.1M Imidazole pH 8, 0.2M $Zn(OAc)_2$	tiny needle clusters
Wizard I	6	18	20% PEG 3K, 0.1M Citrate pH 5.5	needle clusters
Wizard I	20	18	0.4M NaH_2PO_4/1.6M K_2HPO_4, 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	27	18	1.2M NaH_2PO_4/0.8M K_2HPO_4, 0.1M CAPS pH 10, 0.2M Li_2SO_4	wispy needle clusters
Wizard I	30	18	1.26M $(NH_4)_2SO_4$, 0.1M NaOAc pH 4.5, 0.2M NaCl	wispy needles
Wizard I	31	18	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	tiny needle clusters
Wizard I	33	18	2M $(NH_4)_2SO_4$, 0.1M CAPS pH 10.5, 0.2M Li_2SO_4	fiber bundles
Wizard I	39	18	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li_2SO_4	needle clusters
Wizard II	4	18	2M $(NH_4)_2SO_4$, 0.1M Cacodylate pH 6.5, 0.2 NaCl	needle clusters
Wizard II	9	18	2M $(NH_4)_2SO_4$, 0.1M phosphate citrate pH 4.2	wispy needles
Wizard II	35	18	0.8M NaH_2PO_4/1.2M K_2HPO_4, 0.1M NaOAc pH 4.5	tiny needle clusters
Wizard II	38	18	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li_2SO_4	tiny needle clusters

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. The contents of all references, patents and patent applications cited throughout this application are hereby incorporated by reference.